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8-Oxoguanine DNA glycosylase-1 augments pro-inflammatory gene expression by facilitating the recruitment of site-specific transcription factors

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Abstract

Among the insidious DNA base lesions, 8-oxo-7,8-dihydroguanine (8-oxoG) is one of the most abundant, a lesion that arises through the attack by reactive oxygen species on guanine, especially when located in cis-regulatory elements. 8-oxoG is repaired by the 8-oxoguanine glycosylase 1 (OGG1)-initiated DNA base excision repair (BER) pathway. Here we investigated whether 8-oxoG repair by OGG1 in promoter regions is compatible with a prompt gene expression and a host innate immune response. For this purpose, we utilized a mouse model of airway inflammation, supplemented with cell cultures, chromatin immunoprecipitation, siRNA knockdown, real-time PCR, Comet and reporter transcription assays. Our data show that exposure of cells to tumor necrosis factor alpha (TNF-α) altered cellular redox, increased the 8-oxoG level in DNA, recruited OGG1 to promoter sequences and transiently inhibited BER of 8-oxoG. Promoter-associated OGG1 then enhanced NF-êB/RelA binding to cis-elements and facilitated recruitment of Specificity Protein 1 (SP1), transcription initiation factor II-D (TFIID), and phospho-RNA polymerase II, resulting in the rapid expression of chemokines/cytokines and inflammatory cell accumulation in mouse airways. siRNA depletion of OGG1 or prevention of guanine oxidation significantly decreased TNF-α-induced inflammatory responses. Together, these results show that non-productive binding of OGG1 to 8-oxoG in promoter sequences could be an epigenetic mechanism to modulate gene expression for a prompt innate immune response.

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There are no conflicts of interest

Introduction

The genomes of living organisms are continuously exposed to reactive oxygen species (ROS) derived from the normal metabolism, and from patho-physiological processes caused by exposure to physical, chemical or infectious agents. ROS inflict oxidative damage on various macromolecules, including proteins, lipids and nucleic acids (both DNA and RNA) (1). Repair of DNA lesions is crucial for maintaining genomic integrity, while other oxidatively damaged macromolecules undergo degradation (2). One of the most abundant oxidized DNA lesions is 8-oxo-7,8-dihydroguanine (8-oxoG), as guanine has the lowest oxidation potential among the four bases in DNA (3). The repair of 8-oxoG is important because it can mispair with adenine, which results in a G:C to T:A transversion during replication, leading to mutations (4). In mammalian cells the enzyme catalyzing the excision of the 8-oxoG base from the DNA duplex is 8-oxoguanine DNA glycosylase1 (OGG1; human and mouse) (5, 6), which is a functional analog of the *Escherichia coli* Fpg protein (5).

The loss or functional deficiency of OGG1 has been linked to mutagenesis, carcinogenesis and is believed to contribute to the aging process and age-associated diseases (7, 8). Intriguingly, the supraphysiological levels of 8-oxoG in *Ogg1*$^{-/-}$ mice do not impair embryonic development, and the mice have a normal life span and show no marked pathological changes or tumor induction (9–11). Moreover, *Ogg1*$^{-/-}$ mice are highly resistant to inflammation induced by ROS, lipopolysaccharide or allergens. These mice express lower levels of pro-inflammatory chemokines/cytokines (e.g., MIP-1α, TNF-α ,IL-4, IL-6, IL-10, IL-12 and IL-17), so the homing of inflammatory cells to the site of injury is decreased (12, 13). Our previous work showed that decreased OGG1 expression and 8-oxoG repair in the airway epithelium resulted in a lower allergic inflammatory response after ragweed pollen challenge of sensitized mice, as shown by decreased expression of Th2 cytokines, eosinophilia, and airway hyper-responsiveness (14). These results imply that 8-oxoG base and/or OGG1 could play a key role in pro-inflammatory gene expression and inflammatory processes.

It is well-documented that transcriptional regulation of pro-inflammatory genes is highly regulated by ROS-mediated signaling. Previous studies have addressed the role of ROS in the posttranslational modifications of transcription factors (e.g., NF-κB, AP-1) (15–17); however, the possibility that oxidative damage to cis-elements may affect the binding of sequence-specific transcription factors and assembly of the transcription machinery has attracted less attention. Intriguingly, vertebrate genome evolutionarily has a high GC content in the promoter regions of RNA polymerase II (RNA Pol II)-transcribed genes, despite guanine's vulnerability to be oxidized to the mutagenic 8-oxoG and subjected to DNA repair. For instance, a genomic-wide survey revealed that 72% of promoters belong to a class with high CpG content (18). Also, the consensus binding sites for many transcription factors (e.g. Sp1 and NF-κB) are guanine-rich (17, 19). We thus speculate that the high GC content in promoter regions is an advantage for transcriptional regulation due to non-productive binding of OGG1 to 8-oxoG under conditions of oxidative stress.

In the present study we show that TNF-α-induced ROS increased 8-oxoG levels in the genome, including promoter region(s), and also binding of OGG1 to the C-X-C-motif chemokine ligand 2 promoter (mouse and human). Bound OGG1 enhanced *Cxcl-2* expression via facilitating the recruitment of TFIID, NF-κB/RelA, Sp1, and phosphorylated RNA pol II (p-Pol II). OGG1 depletion decreased transcription factor binding to the promoter and TNF-α-mediated innate immune responses (IIR) in a mouse model of airway inflammation. These data strongly suggest a role of OGG1 in transcriptional activation of pro-inflammatory genes in response to oxidative stress.

Materials and Methods

Reagents and antibodies

TNF-á was purchased from PeproTech Inc (Rocky Hill, NJ); N-acetyl-L-cysteine (NAC) Tris, NaCl, EDTA, EGTA, Nonidet P-40, sodium pyrophosphate, glycerophosphate, $Na3VO_4$, NaF, aprotin/leupeptin/PMSF, sodium dodecyl sulfate, and paraformaldehyde were from Sigma-Aldrich (St Louis, MO, USA). Antibodies (Abs) to RelA, Sp1, TFIID, GAPDH and control IgG were from Santa Cruz Biotech (Santa Cruz, CA, USA), and Ab to phosphorylated RNA polymerase II was from COVANCE Biotechnology (Princeton, New Jersey, USA). Anti-OGG1 and anti-Flag Abs were from Epitomics (Burlingame, CA, USA) and Sigma-Aldrich, respectively.

Animals, challenge and evaluation of inflammation

Six- to 8-week-old OGG1-proficient or deficient female BALB/c mice (∼ 20 g, Harlan Sprague-Dawley, San Diego, CA, USA) were TNF-á-challenged intranasally with or without anti-oxidant (AO) pre-treatment. OGG1 depletion was performed as described bellow and AO treatment was achieved by intra-peritoneal injection of NAC (320 mg/kg) plus ascorbic acid (400 mg/kg) 1 h before TNF-á challenge. RNA from the upper one third of lungs was extracted as described previously (20). Expression of inflammation-related cytokines and chemokines was analyzed by plate-based q-PCR arrays (SABiosciences, Valencia, CA, USA) using pooled cDNA from each group as template (n=5). To evaluate inflammation, bronchoalveolar lavage fluids (BALF) were collected 16 h post-challenge, processed, cytospin slides stained with Wright-Giemsa, and the number of neutrophils was counted as we previously described (21). All experiments were performed according to the NIH Guidelines for the Care and Use of Experimental Animals. The protocol used was approved by the University of Texas Medical Branch Animal Care and Use Committee (#0807044A).

Cell cultures

HEK 293 (Human Embryonic Kidney 293) cells were maintained in DMEM high-glucose medium, and MLE-12 (an immortalized type 2 mouse lung epithelial cell line) was cultured in RPMI 1640 medium, per the instructions of the American Type Culture Collection. $Ogg1^{-/-}$ and $Ogg1^{+/+}$ mouse embryo fibroblasts (MEFs) (10) were kindly provided by Dr. Deborah E. Barnes (Imperial Cancer Research Fund, Clare Hall Labs, United Kingdom) and cultured in DMEM/Ham's F-12 medium (22). All media were supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, NJ, USA), penicillin (100 units/ml; GIBCO-BRL, Gaithesburg, MD, USA), and streptomycin (100 μg/ml; GIBCO-BRL). Cells were regularly tested for mycoplasma contamination.

Depletion of OGG1 expression

To deplete OGG1 from mouse lungs, Stealth RNAi™ (Cat# MSS237431; Invitrogen Life Technologies, Carlsbad, CA, USA) was utilized. Under mild anesthesia, parallel groups of mice were treated with Stealth RNAi™ to *Ogg1* (or control RNAi) intranasally, and depletion of *Ogg1* mRNA in airway epithelial cells was determined by real-time PCR (20) using primers purchased from Integrated DNA Technologies (Newark NJ, USA, Cat# Mm.PT.56a.30885470). Small interfering (si)RNA transfection of cultured cells was performed using the N-TER Nanoparticle Transfection System (Sigma-Aldrich) per the manufacturer's instructions. siRNA to down-regulate mouse *Ogg1* (Cat # M-048121-01-005) and human *OGG1* (Cat# M-005147-03-0005) were purchased from Dharmacon (Pittsburg, PA, USA). Depletion of the target proteins was determined by Western blotting.

RNA extraction and real-time PCR analysis

RNA was extracted from mouse lungs, HEK 293 and MLE-12 cells using an RNeasy kit per the manufacturer's instructions (Qiagen, Valencia, CA, USA). Total RNA (1 ìg) was reverse-transcribed using a SuperScript® III First-Strand Synthesis System (Invitrogen Life Technologies). Inflammation-related Cytokines and Chemokines q-PCR Arrays were analyzed as suggested by the manufacturer (SABiosciences). mRNA levels of individual genes in cultured cells were determined using primers purchased from Integrated DNA Technologies : m-*Cxcl-2*: F: 5'- CTCCTTTCCAGGTCAGTTAGC −3', R: 5'-CAGAAGTCATAGCCACTCTCAA −3'; m-*Gapdh*: F: 5'-CTCATGACCACAGTCCATGC-3', R: 5'-CACATTGGGGGTAGGAACAC-3'; h-*CXCL-2*: F: CACACTCAAGAATGGGCAGA-3', R: 5'-CTTCAGGAACAGCCACCAAT-3'; h-*GAPDH*: F: 5'-CTGGAGAAACCTGCCAAGTA-3', R: 5'-TGTTGCTGTAGCCGTATTCA-3'. Real-time-PCR was performed in an ABI7000 thermal cycler. Relative expression levels of target genes were calculated by the ÄÄCt method as we described previously (20).

Reporter and expression constructs

The mouse Cxcl-2 promoter (−571 to +81) was cloned from the MLE-12 genome and inserted into reporter vector pGL4.2 (Promega, Madison, WI, USA) using restriction enzyme sites Kpn I and Bgl II to generate the construct Cxcl-2-Luc. The proximal Sp1 binding site (−109 to −100) deletion mutation was designated as Cxcl-2-Sp1-del-Luc. The inserted DNA was confirmed by sequencing. The plasmid pRL-SV40 encoding Renilla luciferase driven by an SV40 promoter (Promega, Madison, WI, USA) was used as an internal control. The expression plasmid (pcDNA3) encoding IκBα-super repressor (IκB-SR; resistant to phosphorylation by IKK), a mutant form of IκBα (S32A/S36A) was used to block NF-κB shuttling from the cytoplasm to nucleus. The cDNA of IκBSR (∼1000 bp) was cloned into pcDNA3 at the BamH I restriction site. The pCMV-N-Flag-OGG1 expression plasmid encoding N-terminal Flag-tagged human OGG1 was constructed as described in our previous report (23).

Transfection and dual reporter luciferase assay

Transfection was performed using lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Cells were seeded in 24-well plates and incubated in growth medium without antibiotics overnight, then transfected with Cxcl-2-Luc (400 ng/well) and pRL-SV40-encoding Renilla luc (40 ng/well). After 4 h, the medium was replaced with complete medium. Twelve h later, the transfected cells were stimulated with TNF-á for various time intervals (0, 1, 3, 6 h) with or without the ROS scavenger NAC, and then lysed. Firefly and Renilla luciferase activities were measured using the dual luciferase assay system (Promega) with GLOMAX Microplate Luminometer (Promega). The transcriptional activity of *m-Cxcl-2* was represented by the firefly luciferase activity normalized to that of Renilla.

Co-immunoprecipitation and Western blotting

HEK 293 cells (1×10^7) were transfected with a Flag-OGG1 expression plasmid, then stimulated with TNF-á as described above, and lysed in lysis buffer (50 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% Nonidet P-40, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l glycerophosphate, 1 mmol/l $Na3VO_4$, 1 mmol/l NaF, and 20 ìg/ml aprotin/leupeptin/PMSF). Cell lysates were centrifuged at 4°C, 13,000 *g* for 30 minutes and the supernatants were incubated with 30 ìl protein G-Sepharose (Millipore Corporation Billerica, MA, USA) at 4 °C for 1 h. The pre-cleared supernatants were incubated with Ab against Flag for 12 h and then protein G-Sepharose for 3 h with continuous rotation. The

immunoprecipitates were then washed with lysis buffer and resolved by sodium dodecyl sulfate–polyacryamide gel electrophoresis (SDS–PAGE). After the proteins were transferred to nitrocellulose membranes, the membranes were washed with TBST (20 mmol/l Tris base, 500 mmol/l NaCl, 0.05% Tween-20, pH 7.5) and blocked with 10 % non-fat dry milk and then incubated for 1 h with primary Ab(s); at 1:1000 dilution and subsequently with horseradish peroxidase–conjugated secondary Ab(s) at a 1:4000 dilution (SouthernBiotech, Birmingham, AL, USA). The signals were detected using the ECL Plus chemiluminescent detection system (GE Life Sciences, Bucking Hampshire, UK).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously (24) with slight modifications. Briefly, Flag-OGG1-transfected HEK 293 cells or MEF ($Ogg1^{+/+}$ and $Ogg1^{-/-}$) cells were stimulated with TNF-α for 30 min. The cells were harvested and the ChIP assays were performed using Abs against Flag or NF-κB/RelA. ChIP reagents were used according to the recommended protocol from Millipore. 1×10^6 cells were cross-linked with 1% paraformaldehyde and sheared with 10-second pulses using Cole-Parmer's GEX 130 Ultrasonic processor (Vernon Hills, IL, USA) equipped with 2-mm tip and set to 30% of maximum power. One ml of the 10-fold diluted reaction mixture was incubated with or without Abs and then immunoprecipitated with protein A- or G-agarose (Millipore, Corporation Billerica, MA, USA) blocked with salmon sperm DNA. Before adding Abs (Flag, NF-κB/RelA) and agarose beads, one tenth of the dilution was directly subjected to DNA extraction and use as input. The precipitates were washed extensively with washing buffers, de-crosslinked, and subjected to regular or real-time PCR. Primers for amplification were: h-CXCL-2 promoter, F: 5'-ATTCGGGGCAGAAAGAGAAC-3', R: 5'-ACCCCTTTTATGCATGGTTG-3'; *m-Cxcl-2* promoter, F: 5'-GAAGGGCAGGGCAGTAGAAT-3', R: 5'-TGAAGTGTGGCTGGAGTCTG-3'. For regular PCR analyses, 35 cycles were applied to amplification from ChIP products.

Assessment of intracellular ROS levels

Amplex UltraRed (10-acetyl-3,7-dihydroxyphenoxazine; Invitrogen) reagent specifically reacts with H_2O_2 in the presence of horseradish peroxidase to generate a stable product, resorufin. Amplex red assays were carried out as we previously described (20) with minor modification. Briefly, after TNF-α addition (0, 5, 10, 15, 25, 30, 60 min) equal numbers of cells were washed with PBS (pH 7.4), harvested and sonicated (3 × 30 sec) in reaction buffer containing the Amplex UltraRed. Mixtures were incubated for 5 min, cell debries were removed (1 min 13,500 g) and changes in resorufin fluorescence were determined at 560 and 620 nm (excitation and emission, respectively) by using a BioTek FLx800 fluorimeter. To establish the standard curve, increasing concentrations of H_2O_2 (0 to 1000 nM) were used. Resorufin formation in cell extracts was inhibited by addition of catalase (5 U/ml; Sigma–Aldrich).

Assessment of protein cysteine oxidation

To determine whether OGG1 undergoes redox changes DCP-Bio1 reagent (KeraFAST, Inc, Boston MA) was used. Briefly, cells were transfected with FLAG-OGG1 plasmid, and then exposed to TNF-α for 0, 15, 30, 60, and 90 min. Cells were lysed in lysis buffer (50 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% Nonidet P-40, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l glycerophosphate, 1 mmol/l $Na3VO_4$, 1 mmol/l NaF, and 20 ìg/ml aprotin/leupeptin/PMSF) containing 100 μ*M* DCP-Bio1 (25). Cell extracts were clarified and the supernatants were incubated with 30 ìl protein G-Sepharose (Millipore Corporation Billerica, MA, USA) at 4 °C for 1 h. The pre-cleared supernatants were then incubated with Ab against Flag for 12 h and added to protein G-Sepharose for 3 h

with continuous rotation. The immunoprecipitates were washed with lysis buffer and resolved by SDS–PAGE. Proteins were transferred to nitrocellulose membranes, blocked with 5% non-fat dry milk in TBST for 1 hr and then incubated with streptavidin-conjugated horseradish peroxidase for 1 hr. OGG1 reacted with DCP-Bio1 were detected by enhanced chemiluminescence. Band intensities were quantitated by densitometry using ImageJ (ver. 1.44) software (NIH) and percentage changes in levels of oxidative modified OGG1 was calculated using MS Excel.

Protein-protein interaction assays

Physical interaction between OGG1 and transacting factors was analyzed as we described previously (26). Briefly, nickel-nitrilotriacetic acid- (Ni-NTA)-agarose beads (Qiagen Inc) were incubated with His-OGG1 protein (6 pmol) (Cytoskeleton, Inc) in interaction buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20, pH 7.5). After 60 min incubation at 4°C, His-OGG1 bound beads were washed 3 times, and equimolar, non-tagged NF-κB/RelA (6 pmol; Catalog #: H00005970-P02, Human RelA, Abnova) was added. To elucidate the interaction of OGG1 with Sp1, his-tagged Sp1 (His-Sp1, ProteinOne, Inc) was used. His-Sp1 (6 pmol; Cat# P1034) was added to Ni-NTA for 60 min and washed, then non-tagged OGG1 (6 pmol; Cytoskeleton, Inc) was added in interaction buffer. After incubated for 60 min at 4 °C samples were washed, proteins were eluted with Laemmle buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, pH 6.8), then subjected to SDS-PAGE and analyzed by Western blotting.

Assessment of 8-oxoG level

8-oxoG in genomic DNA was assessed by determining the levels of OGG1-sensitive sites using an OGG1 FLARE™ Comet Assay (fCA; Travigen, Gaithersburg, MD, USA) (14, 27). Briefly, MLE-12 cells with or without TNF-α challenge were embedded in agarose, lysed, detergent was removed by washing, and recombinant (r)OGG1 protein was added in the digestion buffer and then the DNA was subjected to alkaline electrophoresis. Fifty cells were evaluated for each data point, using the Comet Assay IV v4.2 system (Perceptive Instruments, Suffolk, UK).

To determine whether TNF-α challenge generates 8-oxoG in the *Cxcl-2* promoter region, MLE-12 cells were challenged with TNF-α for various length of time (0, 15, 30, 60 and 90 min), and genomic DNA was extracted (QIAamp DNA kit, Qiagen, Stanford Valencia, CA). Genomic DNA (20 ng) was subjected to real-time PCR to amplify the proximal region of the *Cxcl-2* promoter, using the primers used in ChIP assays (See Section 9, above).

To examine the 8-oxoG levels in the proximal region of the *Cxcl-2* promoter, 2 μg of genomic DNA from each sample was incubated with or without recombinant OGG1 (rOGG1, Novus Biologicals) in a 100 μl reaction (50 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 1 mmol/l EDTA, 2 mmol/l DTT, 1 mg/ml BSA, and 5 ng rOGG1) at 37 °C for 20 min. After digestion, DNA was subjected to phenol/chloroform extraction, and 20 ng of DNA from each sample was used as a template to amplify the proximal region of the *Cxcl-2* promoter as described above. rOGG1's base excision activity was determined using a 40-mer oligonucleotide containing an 8-oxoG at position 19 and labeled at the 3′ end with Cy5 (5'-AGAGAAGAAGAAGAAGAA /8oxodG/AGATGGGTTATTCGAACTAGC/ 3Cy5Sp/-3'), as we previously described (28).

Statistical analysis

Statistical analysis was performed using Student's *t*-test to analyze changes at the mRNA and protein levels. Data from mouse treatment groups were analyzed by ANOVA, followed by Bonferroni post-hoc analyses for least significant difference. The data are presented as

the means ± the standard error of the mean. Differences were considered to be statistically significant at $P < 0.05$.

Results

OGG1 depletion decreased TNF-α-induced inflammation in mouse lungs

To address the role of OGG1 in the regulation of pro-inflammatory genes, mice expressing OGG1 (proficient) or depleted in OGG1 (deficient) were challenged intranasally with TNF-α. One hour after the challenge, the lungs were excised, RNAs extracted, and cDNAs synthesized and subjected to analysis by real-time PCR arrays for cytokine and chemokine expression. TNF-α challenge upregulated the expression of inflammatory mediators including CXC-motif chemokine ligands (CXCLs), CC chemokine ligands (CCLs), interleukins (ILs) and TNF-α. For instance, *Cxcl-1* mRNA was increased 75.9-fold, and that for *Tnf*-α 23-fold (Fig. 1A). Unexpectedly, OGG1 depletion from the airway epithelium significantly decreased TNF-α-induced pro-inflammatory gene expression. For example, the extent of *Cxcl-1* and *Tnf*-α mRNA induction decreased to 23.6- (by ∼70%) and 2.84-fold (by ∼90%), respectively, compared to that of OGG1-expressing mice (Fig. 1 B). *Ogg1* mRNA expression in airway epithelial cells was decreased by 85 ± 6.3% (data not shown) after application of *Ogg1* Stealth RNAi, similar to what we have shown previously (14). In selected experiments mice were treated with anti-oxidants (AOs; NAC plus ascorbic acid, as described in Materials and Methods). Pre-treatment with AOs significantly decreased the expression of pro-inflammatory mediators after TNF-α challenge (Fig. 1 C), consistent with previous observations (16, 29). AOs alone had no effect on the expression of inflammatory mediators (data not shown).

The chemokines CXCL-1 and CXCL-2 [homologs of human growth-regulated protein alpha (Gro-α) and beta (Gro-β), respectively] are potent attractants of neutrophils, so we further examined their mRNA levels by real time RT-PCR using individual primer pairs. The results not only confirmed our PCR array data (Fig. 1A), but showed even higher increases in mRNA levels for CXCL-1 (281 ±73-fold, Fig. 1D left panel) and CXCL-2 (535 ±73-fold; Fig. 1D, right panel) in TNF-α-challenged lungs. As expected from the robust increase in *Cxcl-1* and *Cxcl-2* expression, TNF-α-challenge induced extensive neutrophila ($>8 \times 10^5$ neutrophils per ml BALF) in the lungs. Intriguingly, OGG1 deficiency significantly decreased *Cxcl-1* (by ∼74%, Fig. 1 D, left panel) and *Cxcl-2* mRNA levels (by ∼80%, Fig. 1 D, right panel), in line with the number of neutrophils in the BALF (Fig. 1E). Pre-treatment of mice with AOs decreased both *Cxcl-1* and *Cxcl-2* mRNA levels (Fig. 1D) and the number of recruited neutrophils in the BALF (Fig. 1E). These results suggested that IIR mediated by TNF-α are ROS- and/or OGG1-dependent.

OGG1 augments TNF-α-induced mRNA expression of CXCL-2

To explore the molecular mechanism by which OGG1, a DNA repair enzyme, modulates the expression of pro-inflammatory genes we utilized a mouse airway epithelial cell (MLE-12) model as in our previous studies (16, 29), with *Cxcl-2* as a representative pro-inflammatory cytokine gene. Addition of TNF-α (20 ng/ml) to the medium resulted in a rapid increase in the level of *Cxcl-2* mRNA, which reached a maximum (>500 ± 44-fold) at 30 min and markedly decreased by 90 min (Fig. 2A). A similar time course of *CXCL-2* (*Gro-β*) gene expression was observed in HEK 293 cells in response to TNF-α treatment (Fig. 2B). Transfection of MLE-12 and HEK 293 cells with siRNA to *Ogg1* (or *OGG1*) lowered the increase in *Cxcl-2* and *CXCL-2* mRNA levels by ∼85% and ∼70%, respectively (Fig. 2C, D), which was proportional to the OGG1 protein levels in siRNA-treated cells (Fig. 2E). Moreover, to confirm OGG1-dependent expression of *Cxcl-2*, we utilized $Ogg1^{-/-}$ and $Ogg1^{+/+}$ mouse embryonic fibroblasts (MEFs). Intriguingly, the increase in *Cxcl-2* mRNA

levels after TNF-α exposure was ∼8 fold higher in *Ogg1*$^{+/+}$ MEFs than in *Ogg1*$^{-/-}$ MEFs (Fig. 2F). The ROS scavenger NAC significantly decreased the TNF-α-induced increase in *Cxcl-2* mRNA (Fig. 2G, H) as well as intracellular ROS levels (Fig 2. I, J) levels, in both cell types after 30 min of exposure, suggesting an essential role of ROS in regulating *Cxcl-2* and *GRO*-β gene expression. Our previous work also showed a ROS-dependent increase in the pro-inflammatory cytokines IL-1β and IL-8 60 min after TNF-α challenge (16). The combined results strongly suggest that ROS generated via TNF-α and OGG1 expression have crucial roles in upregulation of the pro-inflammatory gene *Cxcl-2*.

OGG1 is implicated in transcriptional activation from the CXCL-2 promoter

To determine whether OGG1 benefits the expression of CXCL-2 via transcriptional activation of the *CXCL-2* promoter, we constructed a reporter plasmid by inserting the mouse *Cxcl-2* promoter (−571 to +81) into the vector pGL4.2. Immediately adjacent to the TATA box there are two NF-κB and one Sp1 binding site(s) (Fig. 3A). The proximal Sp1 binding site deletion mutant was constructed by deleting the consensus nucleotides (−109 to −100). Time-course studies in which we utilized the luciferase activity assay revealed an increase in activation of the *Cxcl-2* promoter from 1 h on, and >10-fold after 6 h of TNF-α treatment (Fig. 3B). No significant increase in luciferase activity was detected in HEK 293 cells transfected with empty vector plasmid pGL4.2 at any time after the addition of TNF-α (data not shown).

Overexpression of IκB-SR, an NF-κB repressor, decreased *Cxcl-2* transcription at 6 h post-challenge nearly to the basal level, implying that NF-κB is an essential factor to enhance the transcriptional activation of *Cxcl-2* (Fig. 3C), in line with our previous observations (16). We also examined whether the proximal Sp1 consensus sequence is involved in the activation of the *Cxcl*-2 promoter. A dual reporter assay of Cxcl-2 promoter deleted in its Sp-1 binding site revealed that activation of the mutant *Cxcl-2* promoter reached only ∼50% of that of the wild-type promoter in response to TNF-α exposure (Fig. 3D). These results imply the involvement of Sp1 in the transcriptional activation of *Cxcl-2*. To address whether OGG1 is implicated in activation of the *Cxcl-2* promoter, we downregulated OGG1 expression in HEK 293 cells before transfection with reporter plasmids. In OGG1-depleted cells TNF-α induced significantly less Luc activity than in control siRNA-transfected HEK 293 cells (Fig. 3E). Treatment with NAC before TNF-α addition decreased *Cxcl-2* promoter activation by ∼50% (Fig. 3F), indicating the involvement of ROS. These results are in line with those showing a decrease in *Cxcl-2* mRNA levels in NAC-treated cells (Figs. 2 G and H). Taken together, these results imply that in response to TNF-α-induced ROS, OGG1 plays a role in the transcriptional activation of *Cxcl-2* driven by NF-κB.

OGG1 binds to CXCL-2 promoter and facilitates the recruitment of NF-κB/RelA

To explore whether OGG1 facilitates *Cxcl-2* transcription based on its binding to the promoter, HEK 293 cells were transfected with a Flag-OGG1 expression plasmid and chromatin immunoprecipitation (ChIP) assays were performed. A 30-min treatment with TNF-α was sufficient to induce an impressive increase in the *CXCL-2* mRNA level; therefore, ChIP assays were carried out at 30 min post-challenge. The results summarized in Fig 4A show the amplification of the 282 bp fragment (which is the proximal region of the h-*CXCL-2* promoter, containing Sp1 and NF-κB binding sequences) in the OGG1-associated chromatin precipitate from TNF-α-treated (but not untreated) cells. As a positive control, Ab to NF-κB/RelA also pulled down these promoter sequences from cells treated with TNF-α, whereas the negative controls (without incubation with Flag or NF-κB/RelA Ab) did not show detectable amplification.

To investigate whether OGG1 expression affects the binding of NF-κB/RelA to the *CXCL-2* promoter region, we utilized OGG1-depleted HEK 293 cells as well as *Ogg*1$^{-/-}$ and *Ogg*1$^{+/+}$ MEF cells. ChIP assays were performed using a NF-κB/RelA Ab. siRNA silencing of OGG1 expression decreased the level of NF-κB/RelA associated with the *CXCL-2* promoter in the ChIP product by ∼70% compared to that in control siRNA-transfected HEK 293 cells (Fig. 4B). Additionally, TNF-α treatment induced a < 2-fold increase in NF-κB/RelA-bound *Cxcl-2* promoter in *Ogg*1$^{-/-}$ MEF cells, while there was a nearly 5-fold increase in NF-κB/RelA-pulldown DNA in *Ogg*1$^{+/+}$ MEF cells (Fig. 4C). Taken together, these data suggest that OGG1 interacts with the promoter and facilitates the subsequent recruitment of NF-κB/RelA.

Binding of OGG1 to 8-oxoG in the Cxcl-2 promoter in TNF-α-treated cells is non-productive

In the human and mouse *CXCL-2* promoters, Sp1 (GGGGCGGGGC) and NF-κB (GGGAATTTCC) binding sites are guanine-rich, which predicts the generation of 8-oxoG upon ROS due to TNF-α exposure (Fig. 2I). 8-oxoG is repaired during OGG1-BER (30), and it is conventionally believed that its efficient removal from DNA benefits sequence fidelity of promoter and transcription. To investigate this possibility, we first performed Flare™ Comet assays as described in Materials and Methods. As expected, the 8-oxoG levels in TNF-α-treated MLE-12 cells were significantly higher than those in untreated cells (Fig. 5A). Fig. 5B shows representative comet moment images of DNA with/without recombinant (r)OGG1 (its excision activity is shown in Fig. 5C) digestion from cells exposed to TNF-α for 30 min.

To examine whether 8-oxoG accumulates in the *Cxcl-2* promoter region, we chromatin IP-d genomic DNA using antibody to Flag-OGG1 from ± TNF-α-treated MLE-12 cells at various time points (0, 15, 30, 60 and 90 min). A 240 bp fragment of the *Cxcl-2* promoter that contains Sp1 and NF-κB binding sites was amplified by real-time PCR. Unexpectedly, the amplifiable amount of *Cxcl-2* promoter was increased after 30 and 60 min of TNF-α treatment (Fig. 5D) despite t he oxidative stress (Fig. 2I). To test if 8-oxoG is present in the *Cxcl-2* promoter, we utilized rOGG1 protein to release 8-oxoG from the chromatin IP-d DNA. We observed that OGG1 digestion significantly decreased the amplifiable amount of *Cxcl-2* promoter sequences [assayed after a 15, 30 and 60 min exposure to TNF-α (Fig. 5E)] when compared the amount of the *Cxcl2* promoter fragment from TNF-α un-treated cells. Interestingly, after 90 min of TNF-α exposure the amplifiable amount of *Cxcl-2* promoter sequence was similar to that at time 0 (Fig. 5E). NAC treatment before TNF-α addition decreased 8-oxoG levels in the DNA and promoter region (data not shown). These results imply that 8-oxoG is formed in the proximal region of the *Cxcl-2* promoter, which was not removed by OGG1 in response to TNF-α treatment. To obtain insight into modification of OGG1, which could account for its decreased repair activity, we utilized the DCP-Bio1 reagent (Material and Methods) that specifically reacts with oxidized cysteine (sulfenic acid) residues (25). Results in Fig. 5F, show that OGG1 oxidatively modified at cysteine residue(s) was the highest at 15 min, then gradually decreased at 30 and 60 min after TNF-α addition. Quantitation of band densities showed that DCP-Bio1-reacting OGG1 represent 14.8 ± 4.27 % of total OGG1 at 15 min. Its level decreased to 11.2 ± 1.9 % and 9.7 ± 3.22 % at 30 and 60 min respectively, after TNF addition (Fig. 5 F, right panel). Cysteine modification has been shown to transiently decrease OGG1's repair activity (31, 32), which may explain the observed increase in 8-oxoG level and binding of OGG1 to the proximal region of the *Cxcl-2* promoter (Fig. 4A and Fig. 5D). These data, combined with the ChIP results, suggested that in TNF-α-treated cells OGG1's binding to the promoter is non-productive in terms of its 8-oxoG excision activity, as such activity would be expected to decrease the amount of fragment amplified, rather than increasing it.

OGG1 interacts with transcription factors in TNF-α-treated cells

The results from the above studies showed that OGG1 is non-productively bound to 8-oxoG in the *Cxcl-2* promoter in response to TNF-α treatment (Fig. 5A), raising the possibility that it interacts with proteins in the transcription complex on the *Cxcl-2* promoter. To test this hypothesis, HEK 293 cells were transfected with Flag-OGG1 expression plasmid, followed by treatment with TNF-α, and Co-IP assays were performed. The results showed that a 15-min TNF-α treatment resulted in increased interactions of OGG1 with Sp1 (Specificity protein 1), transcription initiation factor (TF) II-D, and p-RNA-Pol II. Interestingly, an increased interaction between OGG1 and NF-κB/RelA was observed from 30 min on, and lasted up to 60 min after TNF-α addition (Fig. 6A). Given that a 15-min TNF-α challenge was not sufficient to increase *Cxcl-2* mRNA levels (Figs. 3 A and B), these results imply that NF-κB/RelA is indeed a limiting factor for the expression of CXCL-2, as shown previously (16, 29). At 90 min after the addition of TNF-α, all these transcription factors were markedly decreased in the OGG1-associated complex, and coincided with a low *Cxcl-2* mRNA level (Fig. 3 A and B). As shown in Fig. 6B, NAC pre-treatment significantly lowered the interaction of OGG1 with NF-κB/RelA and RNA p-Pol II.

Next, we explored a possible physical interaction between OGG1 and NF-κB/RelA and Sp1. To do so, we carried out protein-protein interactions assays as described in Materials and Methods. Results summarized in Fig. 6C and D, show that OGG1 binds both NF-κB/RelA and Sp1. Quantitation of eluted OGG1 and comparison with input NF-κB indicated a nearly equimolar binding of NF-κB/RelA to OGG1 and OGG1 binding to Sp1 suggesting that these transacting factors are directly interacting with OGG1 protein. From these results and those showing cysteine oxidation in OGG1 (Fig. 5F) one may propose that oxidatively modified OGG1 binds to promoter sequences and interacts with transacting factors in TNF-α-exposed cells. To test this hypothesis we examined whether NF-kB/RelA and/or Sp1 interacts with cysteine sulfenic acid-containing OGG1. To do so, Flag-OGG1 expressing cells were exposed to TNF-α, lysed in the presence of DCP-Bio1 and Co-IP assays were performed using NF-κB/RelA or Sp1 Abs. As shown on Fig. 6E,F, OGG1 was present both in NF-κB/RelA and Sp1 immune complex. More importantly, OGG1 was modified at cysteine (Fig. 6E,F) suggesting that oxidative modification is required for its physical interaction(s) with trans-acting factors in TNF-α-exposed cells.

Discussion

ROS generated by generated by biological, chemical, physical agents, ligand-receptor interaction(s) or combinations thereof induce cell signaling and modify proteins, lipids and nucleic acids. 8-oxoG is the most common oxidative lesion in DNA because guanine has the lowest ionization potential among the DNA bases (1, 4). 8-oxoG preferentially accumulates in the guanine-rich genomic regions, including those in the enhancer/promoter regions of genes. It is expected that 8-oxoG is repaired in order to avoid mutations and to maintain the sequence fidelity of promoters for efficient binding of sequence-specific transcription factors. In this study, we made unexpected discoveries suggesting that an association of OGG1 with proximal promoter region(s) and its interaction with Sp-1, NF-κB, TFIID and p-RNA Pol II play a fundamental role in TNF-α-induced expression of pro-inflammatory chemokine(s).

These results are highly unexpected, as OGG1 is thought to be a canonical DNA BER enzyme for the removal of 8-oxoG (and Fapy G) due to its DNA glycosylase/AP-lyase activity. The repair activities initiated by OGG1 are complex, and modulated by post-translational modifications, including phosphorylation (33) and acetylation (23), as well as by its interactions with other repair and non-repair proteins (34). Recent reports also showed that redox changes affecting cysteines significantly decreases OGG1's activity (31, 32).

Binding of OGG1 to 8-oxoG-containing in promoter regions that plays a role in the enhancement of transcription from target genes has never been described.

We have previously documented that TNF-α increases intracellular ROS and genomic 8-oxoG levels, with a peak increase at 15 min (16, 35). We also showed that TNF-α-induced phosphorylation of NF-κB/RelA at Ser^{276} (p-NF-κB/RelA) and its nuclear translocation are tightly associated with ROS signaling, and that inhibition of TNF-α-induced ROS blocks the activities of kinases and decreases NF-κB/p-RelA levels (16, 35). Importantly, we showed that p-NF-κB/RelA stable enhanceosome formation with p300 and p-RNA Pol II and binding to the IL-8 promoter are ROS-dependent, and that the chemically unrelated antioxidants dimethyl sulfoxide, NAC, and/or vitamin C decreased TNF-α-induced IL-8 expression (16, 35, 36).

In the present study we document that TNF-α induced a robust oxidative stress-dependent expression of CXCL and CCL pro-inflammatory mediators and neutrophilia in mouse lungs, which was inhibited by AOs (NAC plus ascorbic acid). Intriguingly, the TNF-α challenge-induced IIR was nearly prevented by OGG1 depletion in the lung epithelium. Likewise, AOs or siRNA to *Ogg1* mRNA significantly decreased pro-inflammatory gene expression in cultured human and mouse cells.

TNF-α has been shown to signal via distinct cell surface receptors, TNFR-1 and TNFR-2 (37, 38). For example, upon binding to TNFR-1, the liganded receptor aggregates to serve as a scaffold to sequentially recruit death domain (DD)-containing adaptor proteins – TNFR-1-associated-DD (TRADD), TRADD-associated factor 2, and the receptor- interacting protein (39). The activated complex in turn recruits kinases, including mitogen-activated protein kinases (MAPK) and IκB kinase, and induces ROS for post-translational modification and activation of transcription factors. Therefore, the effects of Ogg1 depletion on the TNF-α-induced IIR are extremely intriguing, while the effect of AOs may be explained by a lack of ROS signaling.

Taking into consideration the similar inhibitory impact of ROS scavengers [in this and previous studies (16, 35)] and of OGG1 depletion on the IIR and the lack of known interactions of OGG1 with TNF-α signaling intermediates, we speculate that the observed phenomena could be associated with oxidative damage to guanines and lack of OGG1 BER of 8-oxoG in the promoter region(s). It is documented that a large number of promoters driving the transcription of redox-sensitive genes have cis-regulatory elements containing runs of guanines (e.g., NF-κB-and Sp1-binding sites) (17, 19). Thus it is reasonable to expect that oxidative damage to guanines and BER of 8-oxoG will hamper the binding of transcription factors to their DNA consensus sequences.

Our results from real-time PCR analysis of the *Cxcl-2* promoter showed that 8-oxoG level had increased transiently suggesting that BER of 8-oxoG did not initiate immediately after TNF-α exposure. These data imply that 8-oxoG in the promoter region appears not to be an obstacle to the binding of transcription factors. Indeed, substitution of the 5’ guanine in guanine runs for 8-oxoG has no significant effect on Sp1 binding *in vitro* (40). Another study showed that a 5’ 8-oxoG in a guanine run actually increased NF-κB/p50 binding to its consensus DNA sequence, while the substitution of later guanines for 8-oxoG decreased p50 binding (41). Binding of Sp1, NF-κB, etc. to 8-oxoG-containing promoters could have significance, as under oxidative stress conditions in a double helix, containing a π-stacked array of heterocyclic base pairs is favorable for the migration of charge over molecular distance, which results in the selective oxidation of 5’ guanine in runs of guanines (42, 43). On the other hand, our data strongly suggest that a TNF-α-induced ROS burst leads to oxidation of guanine, and OGG1 as well as a non-productive binding of OGG1 to its

substrate. Consequently, this complex is beneficial for the binding of transcription factors, and mRNA synthesis. Indeed, OGG1 depletion significantly decreased TNF-α-induced gene expression.

Our ChIP analysis showed that OGG1 binds to the *Cxcl-2* promoter, and it appears that its presence is essential for the recruitment of transcription factors. To prove this point, we show that amplification by real-time PCR from a 240 bp (MLE-12 cells) and a 282 (HEK 293 cells) bp fragment of the *Cxcl-2* promoter was increased in OGG1-expressing cells after TNF-α addition, compared to amplification of those from untreated cells. Our data also showed that TNF-α-induced oxidative stress increases 8-oxoG levels in the promoter, as digestion of extracted DNA with rOGG1 before real-time PCR significantly decreased amplification from the *Cxcl-2* promoter. These results imply that after TNF-α addition OGG1 still binds 8-oxoG in the *Cxcl-2* promoter, but its excision activity is inhibited. The mechanism by which OGG1's BER activity is inhibited is not known, but we speculate that TNF-α-induced cellular redox changes may indeed hamper OGG1's 8-oxoG glycosylase/AP lyase activity. In support of this possibility, elegant studies showed that oxidation of cysteine residues, of which eight are present in both human and mouse OGG1, could be responsible for its decreased 8-oxoG excision activity (31, 32). Cysteine sulfenic acid is a well established oxidative stress-induced modifier of enzymatic activities, disulfide bond formation, and is key in protein folding (44). To obtain insight into oxidative modification of OGG1 at cysteine(s) in TNF-α exposed cells we utilized DCP-Bio1 reagent that specifically reacts with cysteine sulfenic acid (25). We observed that a significant portion (∼15%) of OGG1 contained cysteine sulfenic acid, suggesting that OGG1 bound to 8-oxoG in G-rich promoter sequences is inactivated via cysteine oxidation. The direct interaction of OGG1 with transacting factors supports our co-IP data and our hypothesis of OGG1's role transcriptional initiation of genes.

It may thus be suggested that OGG1 non-productively binds 8-oxoG in the promoter sequence under oxidizing conditions such as those induced by TNF-α, and promotes transcriptional activation of pro-inflammatory genes. The reestablishment of normal cellular redox status [by ∼90 min, as shown here and in our previous studies (16)] coincided with a sharp decrease in *Cxcl-2* mRNA levels after TNF-α challenge. Therefore, we speculate that OGG1's glycosylase/AP lyase activity is reestablished at this time, and DNA repair intermediates (e.g., apurinic/apyrimidinic sites) formed during the BER of 8-oxoG in the promoter could be associated with the loss of (defects in) cis-elements and dislocation of transcription factor and thus termination of transcription.

Our data also show that OGG1 depletion decreased pro-inflammatory mediator expression in cultured cells and the IIR in mice after TNF-α challenge. Moreover, TNF-α-induced binding of NF-κB/RelA to the *Cxcl-2* promoter was significantly lower in *Ogg*1$^{-/-}$ than in *Ogg*1$^{+/+}$ cells, and Sp1, TFIID, and p-RNA Pol II were present in the OGG1 IP complex. We also show that OGG1 depletion decreased TNF-α-induced binding of NF-κB/RelA to the *Cxcl-2* promoter. These results are consistent with the finding that we have made by using *in vitro* binding assays. We found a direct and equimolar interaction between OGG1 and NF-kB as well as Sp1, which is in line with presence of OGG1 in IP complex. We furthered these observation by showing that NF-κB and Sp1 interacts with a glycosylase inactive cysteine-modified OGG1. The significance of these intriguing observations is yet to be uncovered, and identification of the OGG1 amino acid residues that interact with Sp1 and NF-κB/RelA will be the focus of future investigations.

Finally, our study identified OGG1-dependent expression of *Cxcl-2* (the homolog of human *GRO*-β), which is the most highly expressed among an array of chemokines and cytokines. A proposed role of OGG1 in transcriptional initiation of pro-inflammatory mediators is

shown in Fig. 7. Expression of CXCL-2 may be induced by oxidative stress, chemical and physical agents; and viral, bacterial, fungi infections; or ligand-receptor interactions and is chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells. Also, increased expression of CXCL-2 is characteristic of senescent cells, aged tissues and has been associated with chronic inflammatory states, such as obesity, aging processes, and age-associated diseases including diabetes (type 1 and 2), Alzheimer's disease and others. Moreover, enhanced IL-8 expression has been shown to promote cancer progression, and is a poor prognostic indicator in therapy of a variety of human malignancies. All inflammatory processes in acute and chronic diseases are associated with an increased oxidative state and formation of 8-oxoG in DNA, especially in guanine-rich promoters. Although it needs to be proven, we propose that continuous formation of 8-oxoG and OGG1 binding to promoter sequences could stimulate chronic inflammatory processes and could be an epigenetic mechanism to modulate gene expression in response to oxidative stress. In support of this hypothesis, mice lacking OGG1 activity show increased resistance to inflammation induced by oxidative stress, lipopolysaccharide endotoxins, and various allergenic proteins (12, 13). Given the rate-limiting role of OGG1 in the expression of *C-X-C* chemokines and TNF-α itself, it could be proposed that modulation of OGG1 activity might be a therapeutic target in the resolution of inflammatory processes that impair organ integrity and promote aging processes, as increased OGG1 activity would be expected to decrease the expression of pro-inflammatory genes and recruitment of inflammatory cells.

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List of Abbreviations

8-oxoG	8-oxo-7,8-dihydroguanine
AOs	anti-oxidants
BER	base excision repair
ChIP	chromatin immunoprecipitation assays
CXCL-2	CXC-motif chemokine ligand-2 *Cxcl-2*,gene or mRNA encoding CXCL-2 (as indicated)
CCL	CC chemokine ligand
CA	Comet assay
fCA	Flare Comet assay
OGG1	8-oxoguanine DNA glycosylase-1 protein
OGG1	human 8-oxoguanine DNA glycosylase-1 gene/mRNA

Ogg1	mouse 8-oxoguanine DNA glycosylase-1 gene/mRNA
ROS	reactive oxygen species
RNA Pol II	RNA polymerase II
p-RNA pol II	phosphorylated RNA polymerase II
Sp1	Specificity Protein 1
TFIID	transcription initiation factor *II-D*

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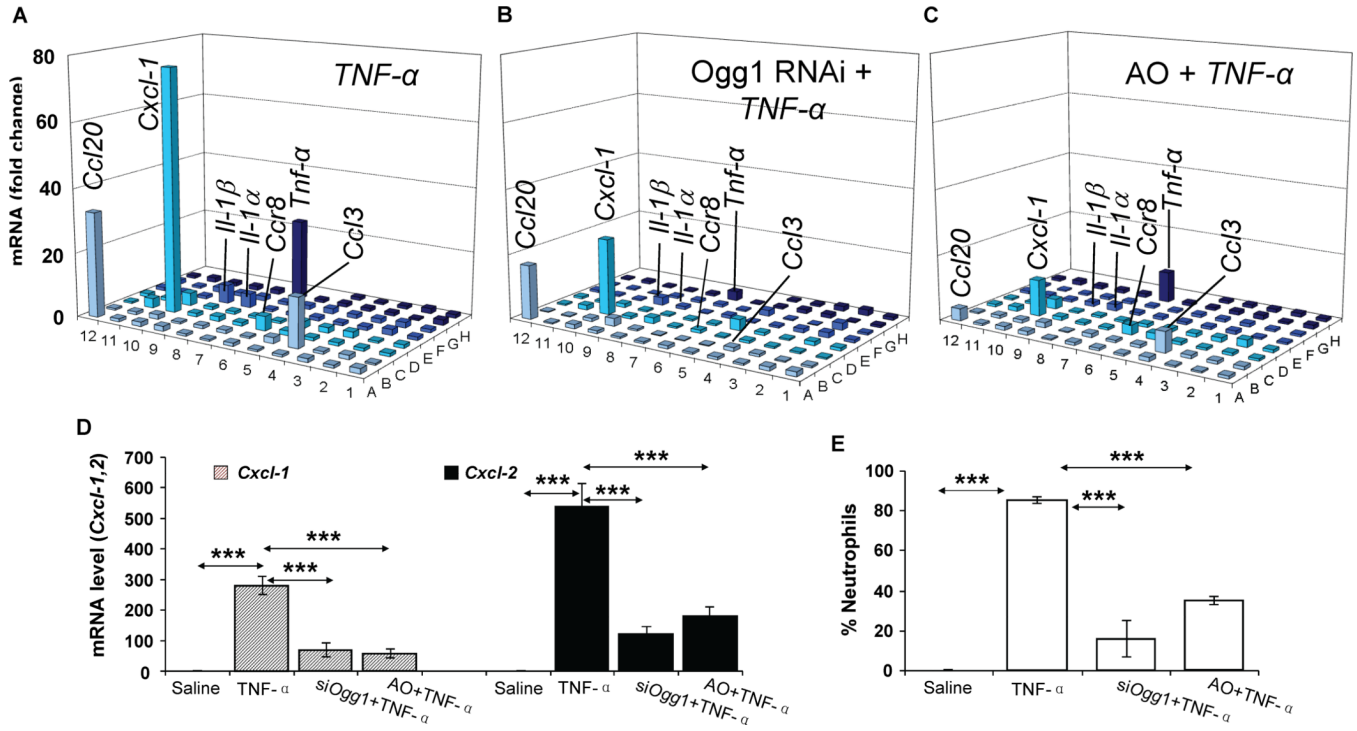

FIGURE 1.

Effect of OGG1 on expression of cytokines and chemokines induced by TNF-á. **A**) TNF-á-induced expression of mRNAs for pro-inflammatory mediators. **B**) OGG1 deficiency decreases TNF-á-induced expression of pro-inflammatory chemokine and cytokine mRNAs. **C**) Effect of AO pre-treatment on TNF-á-induced expression of pro-inflammatory mediators. In A, B and C, groups of mice proficient or deficient in expressing OGG1 in the airway epithelium were challenged intranasally with TNF-á. One hr later, lungs were excised and RNA was extracted. Pooled cDNA from each group (n = 5) was used as a template to perform plate-based inflammation-related PCR arrays. **D**) Changes in *Cxcl-1* (left panel) and *Cxcl-2* (right panel) mRNA levels as determined by real-time PCR in individual lung RNA extracts. OGG1 depletion and AO pre-treatment are described in Materials and Methods. **E**) Effect of OGG1 depletion and AO on the number of neutrophils in BALF of TNF-α-challenged mice. Mice were TNF-α-treated and lavaged as in the Materials and Methods, at 16 h. The percentage of neutrophils was determined as in Materials and Methods. n= 5; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

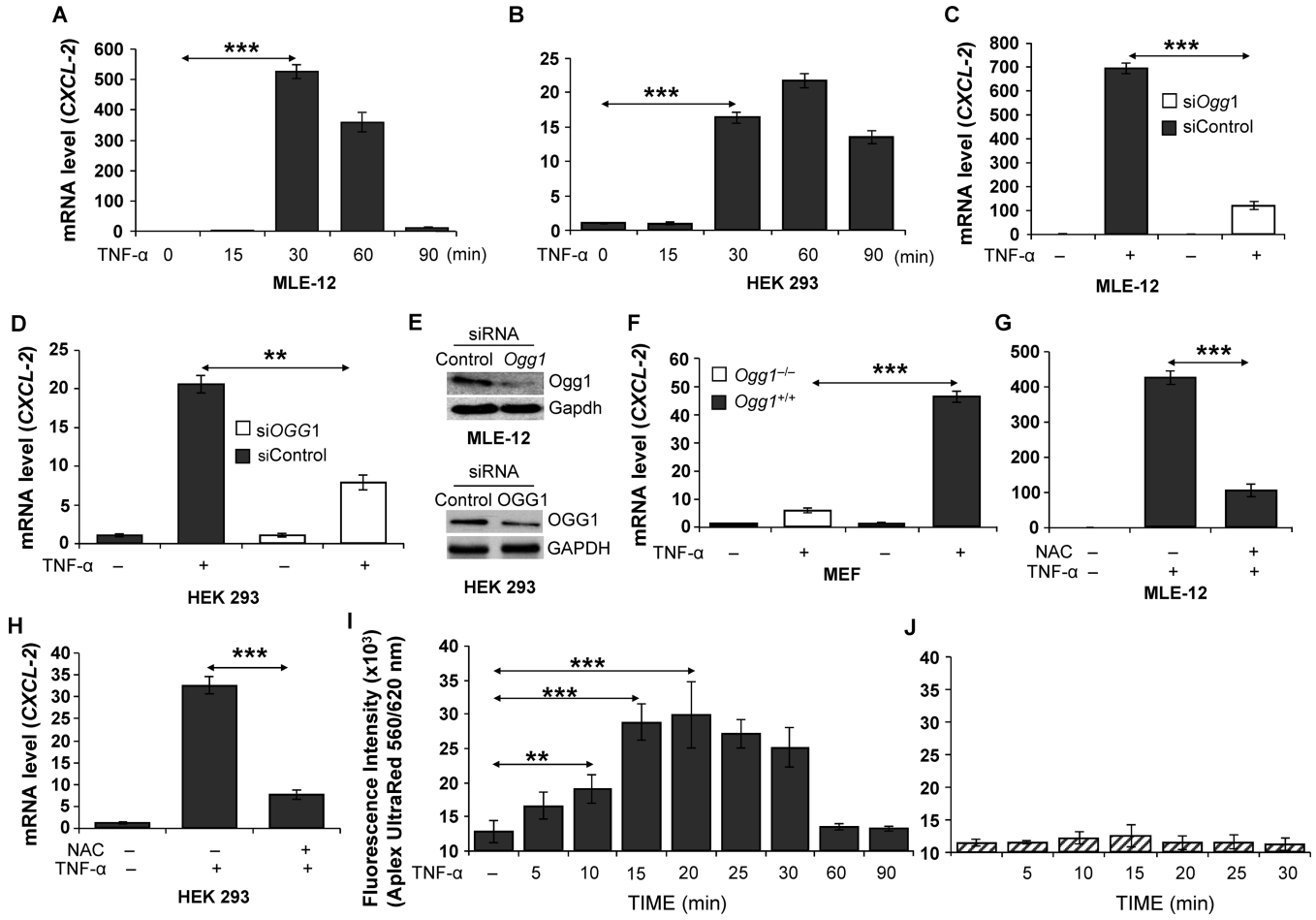

FIGURE 2.

TNF-α-induced expression of *Cxcl-2* mRNA is decreased in OGG1-depleted cells. **A,B**) Time course of *Cxcl-2* mRNA expression upon TNF-α exposure. MLE-12 (A) and HEK 293 (B) cells were TNF-α-treated for various lengths of time, RNA was extracted and real-time PCR performed. **C,D**) OGG1 depletion by siRNA lowers the increase in *Cxcl-2* mRNA levels upon TNF-α treatment. OGG1-depleted and control cells (MLE-12, C; HEK 293, D) were exposed to TNF-α for 30 min, RNA was extracted and real-time PCR was performed. **E**) OGG1 protein levels after siRNA silencing of *Ogg1*. MLE-12 (upper panels) and HEK 293 (lower panels) cells were transfected with siRNA to *Ogg1*, lysed and Western blot analyses performed. **F**) Mouse embryonic fibroblast (MEF) cells lacking OGG1 activity express low levels of *Cxcl-2* mRNA after TNF-α exposure. $Ogg1^{+/+}$ and $Ogg1^{-/-}$ MEF cells were treated with TNF-α for 30 min, RNA was extracted and real-time PCR was performed. **G,H**) The antioxidant NAC decreases TNF-α-induced expression of the *Cxcl-2* gene. MLE-12 (G) and HEK 293 (H) cells were challenged with TNF-α for 30 min ± NAC, RNA was extracted and real-time PCR performed. I) Kinetic changes in cellular ROS levels in TNF-α-exposed cells as determined by Amplex UltraRed assays. J) NAC pre-treatment decreases ROS levels in TNF-α-exposed cells. n = 3–5 **, $p<0.01$; ***, $p<0.001$

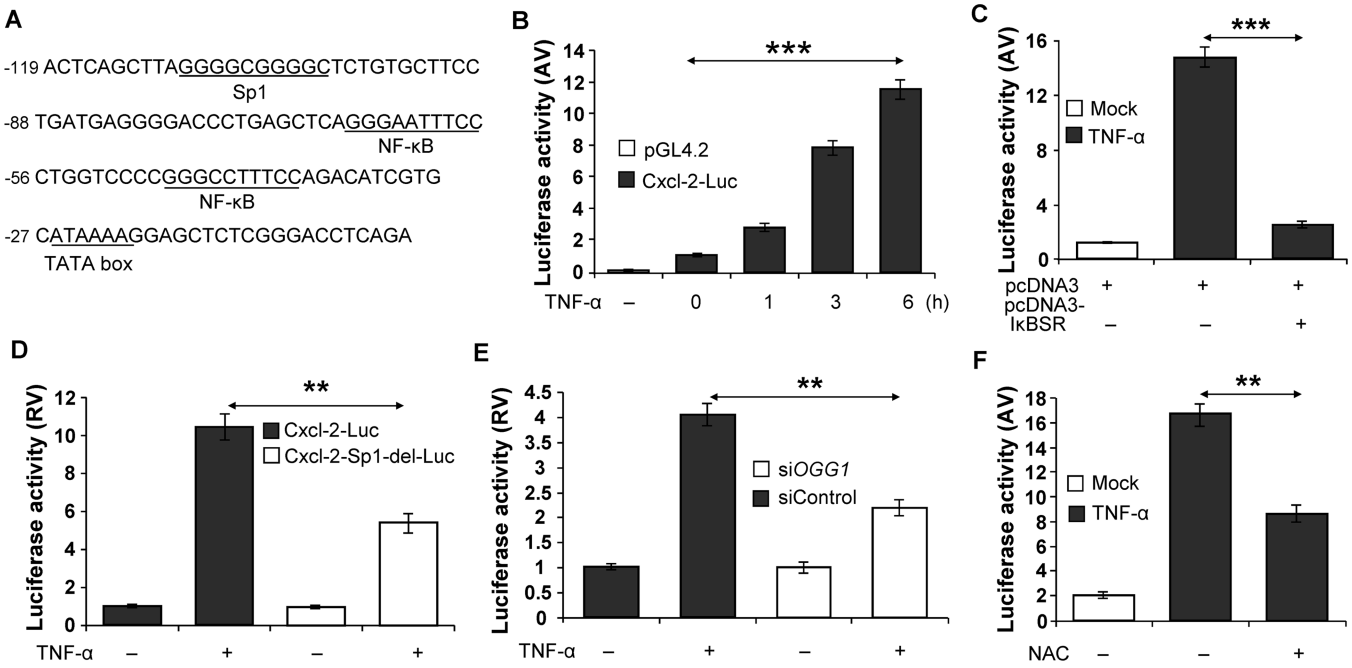

FIGURE 3.

OGG1 expression enhances TNF-α-induced activation of the *CXCL-2* promoter. **A**) Diagram of the proximal region of the mouse *Cxcl-2* promoter (containing the TATA box, two NF-κB and one Sp1 binding sites). **B**) TNF-α exposure activates the *Cxcl2* promoter. HEK 293 cells were transfected with a reporter plasmid (Cxcl-2-Luc) or control vector (pGL4.2) and then challenged with TNF-α for the time intervals indicated. Dual reporter assays were performed as in Materials and Methods. **C**) Activation of the *Cxcl-2* promoter by TNF-α is inhibited by an NF-κB superrepressor (IκBα-SR). HEK 293 cells were transfected with the reporter plasmid Cxcl-2-Luc with or without overexpression of IκBα-SR (pcDNA3-IκBα-SR), then challenged ± TNF-α for 6 h. **D**) Deletion of the Sp-1 consensus sequence decreases TNF-α-induced *Cxcl-2* promoter activation. HEK 293 cells were transfected with report plasmids Cxcl-2-Luc or Cxcl-2-Sp1-del-Luc, and then mock or ±TNF-α-challenged for 6 h. Luciferase activity in cells without TNF-α challenge was taken as 1. **E**) OGG1 depletion inhibits TNF-α-induced *Cxcl-2* promoter activation. HEK 293 cells were transfected with siRNA to *OGG1* (or control), and transfected with reporter plasmid. After a 12 h recovery, cells were treated ± TNF-α for 6 h. Luciferase activity in cells without TNF-α challenge was taken as 1. **F**) Antioxidant pre-treatment decreased TNF-α-induced *Cxcl-2* promoter activation. HEK 293 cells were transfected with the reporter plasmid Cxcl-2-Luc, treated with NAC as in Materials and Methods and then mock- or TNF-α-challenged for 6 h. AV: absolute value, RV: relative value. n = 4–6 **, $p<0.01$; ***, $p<0.001$.

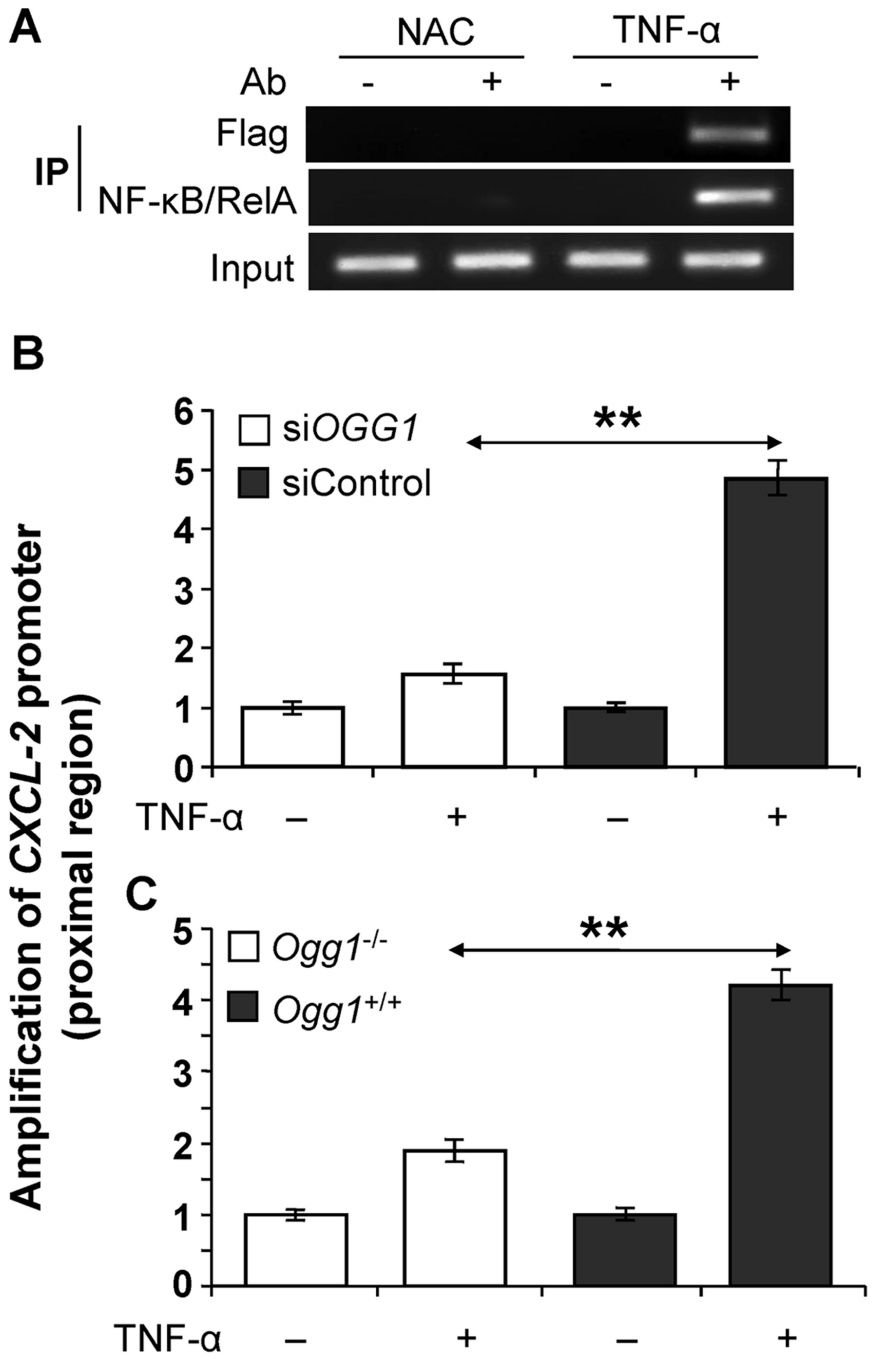

FIGURE 4.

OGG1 binds to the *CXCL-2* promoter and facilitates NF-κB/RelA recruitment. **A**) TNF-α increases binding of OGG1 and NF-κB/RelA to the *CXCL-2* promoter. HEK 293 cells were transfected with Flag-OGG1 plasmid, then treated ± TNF-α for 30 min. ChIP assays were performed using Abs against Flag and NF-κB/RelA. The pulled-down *CXCL-2* promoter was detected by PCR amplification and agarose electrophoresis (amplification from samples without Ab incubation served as negative controls). A representative set of experiment is shown out of three. **B**) OGG1 depletion decreases the association of NF-κB/RelA with the *CXCL-2* promoter. HEK 293 cells were transfected with siRNA (or control siRNA) to

OGG1, then treated ± TNF-α for 30 min. **C**) Lack of OGG1 activity in MEFs hampers the binding of NF-κB/RelA to *Cxcl-2* promoter sequences. *Ogg1*$^{-/-}$ and *Ogg1*$^{+/+}$ MEFs were treated ± TNF-α for 30 min. In B and C, ChIP assay was performed using Ab to NF-κB/RelA. Quantitative amplification of the *CXCL-2* promoter from the ChIP products of different cells was compared by real-time PCR. Amplification from the ChIP products was normalized to that from input genomic DNA, and the value for untreated cells was taken as 1. n = 3–4 **, $p<0.01$

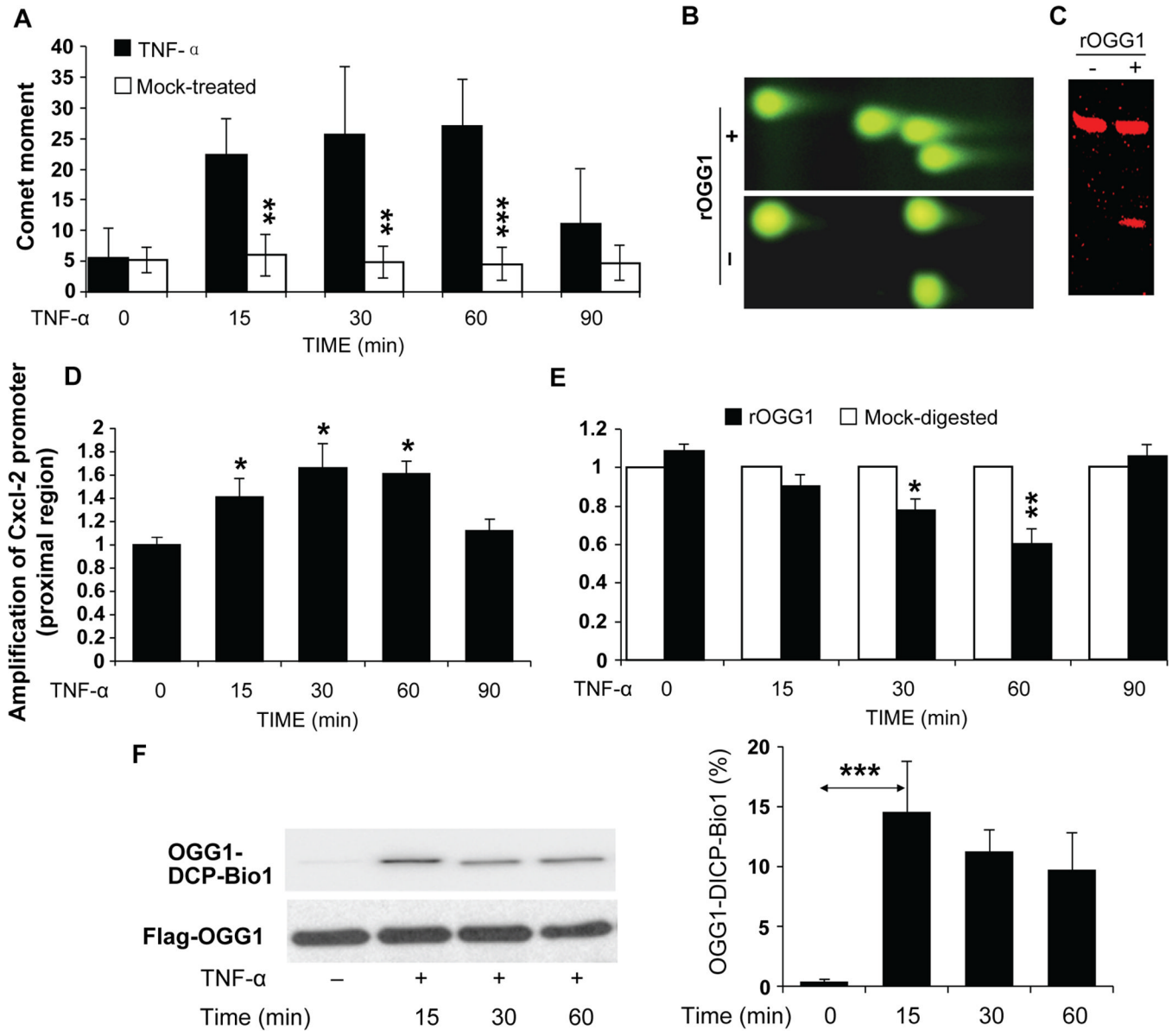

FIGURE 5.

TNF-α treatment increases the integrity of the *Cxcl-2* promoter. **A**) Accumulation of 8-oxoG in genomic DNA in TNF-α treated cells. MLE-12 cells were challenged ± TNF-α for 0, 15, 30, 60 and 90 min. OGG1 FLARE™ Comet Assays were performed as in Materials and Methods. **B**) Representative images show comet moments of DNA with or without rOGG1 digestion (after 30 min of TNF-α exposure). **C**) 8-oxoG excision activity of rOGG1. OGG1's activity was determined using Cy5-labeled 8-oxoG probe (Materials and Methods). **D**) The amplifiable amount of *Cxcl-2* promoter is increased in response to TNF-α challenge. MLE-12 cells were exposed to TNF-α for time intervals indicated. Genomic DNA was extracted and real-time PCR was performed to determine amount of proximal 240 bp long region of *Cxcl-2* promoter. **E**) 8-oxoG accumulates in *cxcl-2* promoter. MLE-12 cells were exposed to TNF-α as in legend to A and the extracted DNA was digested with rOGG1 then 240 bp long region of *Cxcl-2* promoter was amplified by real-time PCR. **F**) Oxidative modifications at cysteines of OGG1 in TNF-α-exposed cells as shown by DCP-Bio1 a

sulfenic acid reacting reagent (left upper panel). Right panel shows percentage of oxidatively modified OGG1 at cysteine(s). n = 3–4, *p<0.05; **, p<0.01; ***, p<0.001.

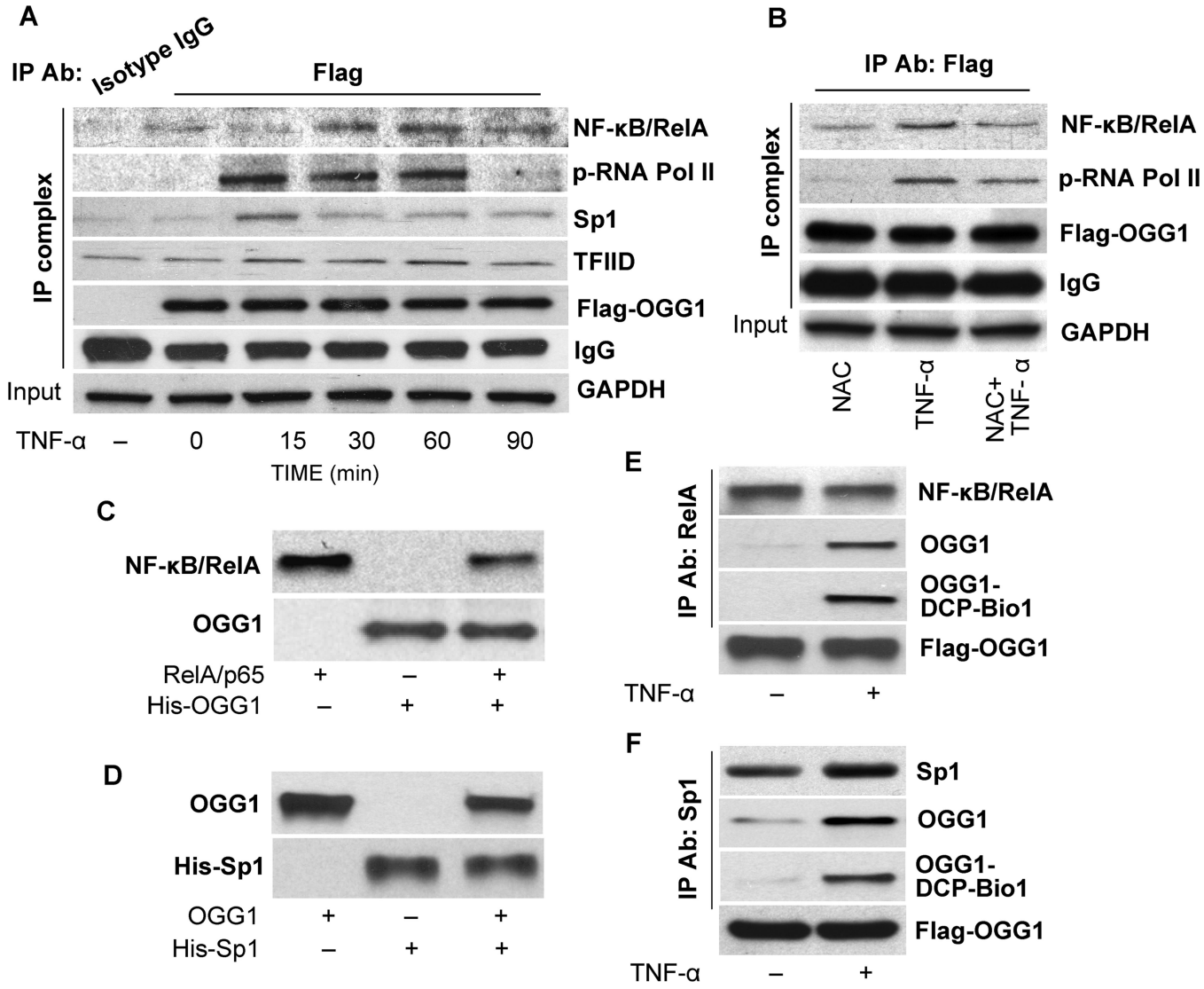

FIGURE 6.

OGG1 interacts with general and site-specific transcription factors. **A**) Interactions of OGG1 with NF-κB/RelA, p-RNA Pol II, Sp1 and TFIID in response to TNF-α challenge. HEK 293 cells were transfected with a Flag-OGG1-expressing plasmid and 24 h later cells were treated with TNF-α for the indicated time intervals. Co-IP was performed using an Ab against Flag. **B**) Anti-oxidant decreased the interaction of OGG1 with NF-κB/RelA and p-Pol II. HEK 293 cells were transfected with the Flag-OGG1-expressing plasmid, and subjected to TNF-α challenge for 30 min. Co-IP was performed to analyze the interaction of OGG1 with NF-κB/RelA and RNA p-Pol II. Shown are representative results of three independent experiments. **C**) Physical interaction between NF-κB/RelA and OGG1. His-OGG1 was immobilized to NTA-agarose beads, washed and then incubated with equimolar non-tagged RelA in interaction buffer for 30 min (Materials and Methods). Bound proteins were eluted and analyzed by Western blotting. **D**) Protein-protein interaction between OGG1 and Sp1. Assays were carried out as in legend to C except His-Sp1 was NTA-agarose-immobilized. **E**) NF-κB/RelA interacts with oxidatively modified OGG1 at cysteine. Flag-OGG1 expressing cells were TNF-α exposed and lysed in buffer containing DCP-Bio1. Co-IP was performed using Ab to NF-κB/RelA. OGG1 associated with RelA

was analyzed for cysteine oxidation (Materials and Methods). **F**) Oxidatively modified OGG1 at cysteine(s) interacts with Sp1. Flag-OGG1 expressing cells were TNF-α exposed and lysed in buffer containing DCP-Bio1. Co-IP was performed using Ab to Sp1. OGG1 associated with Sp1 was analyzed for cysteine oxidation (Materials and Methods). n = 3–4.

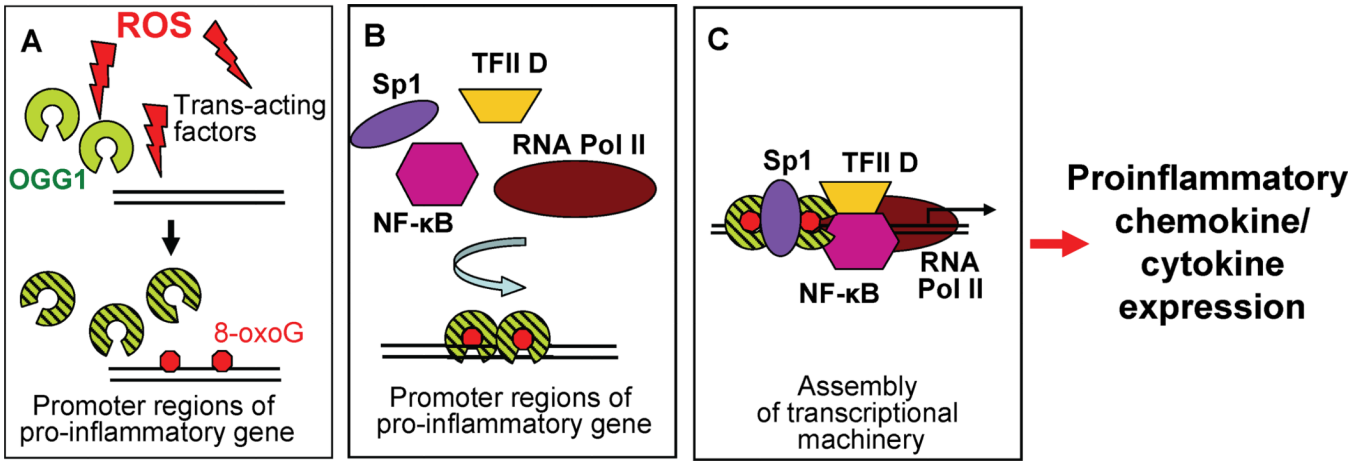

FIGURE 7.

A model of OGG1-driven transcriptional initiation of pro-inflammatory mediators. A) Oxidative modification to guanine and OGG1 as well as activation of trans-acting factors by ROS. B) Non-productive binding of OGG1 to 8-oxoG in promoter region of pro-inflammatory gene(s). C) Assembly of transcriptional machinery facilitated by OGG1.